Discordance of SSA/Ro and SSB/La Cellular Antigens in Synchronized Cells

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SSA/Ro and SSB/La are soluble cellular proteins to which antibodies are frequently produced in patients with Sjögren's syndrome and systemic lupus erythematosus. In this investigation, we examined anti-SSA/Ro and anti-SSB/La staining patterns on synchronized WiL2 cells and mixed lymphocyte culture cells using monospecific antisera. In addition to its presence in the nucleoplasm, the SSB/La antigen was highly concentrated in the nucleolus of cells during the late G_1 and early S phase and is thus cell cycle-related. In contrast, the SSA/Ro antigen was found to be independent of cell cycle, showing a nuclear speckled pattern in all phases. Blocking experiments indicated that free SSB/La is responsible for the nucleolar staining, whereas the combination of both SSA/Ro and SSB/La determines the nucleoplasmic speckled staining pattern. *J Invest Dermatol 87:504–509, 1986*

A prominent finding in patients with connective tissue diseases is an alteration in immunologic responses, including the production of autoantibodies to a variety of antigens. Two such antigens are SSA/Ro and SSB/La, soluble cellular proteins whose exact location, nuclear and/or cytoplasmic, has yet to be clearly determined [1]. Antibodies to these proteins are most commonly found in patients with Sjögren's syndrome (sicca complex) and systemic lupus erythematosus (SLE). Additionally, anti-SSA/Ro antibodies can be detected in patients with so-called ANA-negative SLE, neonatal LE, subacute cutaneous LE, and more recently, complement component C_2 deficiency [2–5].

Antibodies to SSA/Ro and SSB/La coexist frequently in these patients [6]. This situation is comparable to the co-occurrence of antibodies to Sm and nRNP [7] and suggests a close relationship between the antigens, perhaps due to shared RNA [8–10].

Recently in cell synchronization studies, we found that the SSB/La antigen is present in the nucleoplasm of cells in the resting phase; it becomes highly concentrated in the nucleolus in the late G_1 and/or early S phase, and then returns to the nucleoplasm in late S, G_2, and M phases [11]. In this paper we confirm our previous work on the expression of SSB/La antigen and extend our data to a comparison with the subcellular location of SSA/Ro at different phases of the cell cycle. The 2 antigens are shown to be expressed at different phases of the cell cycle, and at different intracellular locations during late G_1 to early S phase; however, competition data suggest some antigenic relationship between them.

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Abbreviations:

ELISA: enzyme-linked immunosorbent assay
FITC: fluorescein isothiocyanate
MEM: minimal essential medium
PBS: phosphate-buffered saline
SLE: systemic lupus erythematosus

MATERIALS AND METHODS

Cell Culture WiL2 human diploid B-lymphoblastoid cells were maintained in suspension culture at 37°C in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum, MEM nonessential amino acids, MEM vitamins, sodium pyruvate, 2 mM glutamine, and antibiotics (Gibco, Buffalo, New York). The cells were subcultured every 3 days at a concentration of 2.0×10^5/ml, except during the synchronization experiments.

To determine DNA synthesis and degree of mitosis, 1.0-ml aliquots of cell suspensions containing 2.0×10^5 cells/ml were pulsed with 10 μCi/ml [^{3}H]thymidine (New England Nuclear) for 30 min. The cells were then washed twice with phosphate-buffered saline (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.4). Cytocentrifugation spreads of these cells were prepared and fixed in acetone for 10 min at room temperature. Processing slides for autoradiography included immersing them in Kodak NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, New York), drying at room temperature, and incubating for 7 days. Slides were then developed in Kodak developer 19, fixed in Kodak fixer, and stained with Giemsa. The percentages of labeled and mitotic cells were expressed as labeled index and mitotic index.

Synchronization of WiL2 Cells Two different methods of synchronization were used to study the distribution of the SSA/Ro and SSB/La antigens in the various phases of the cell cycle. In the first method, WiL2 cells were synchronized by starvation or density-dependent arrest as described previously [12]. After seeding at a concentration of 2×10^5/ml, cell count and viability were determined every day for 7 days using the 0.05% trypan blue exclusion method without addition of new medium. On the sixth day of culture, 60% of the cells remained viable and less than 1% and 0.1% of the cells were showing DNA synthesis and mitosis, respectively (Fig 1). Protein synthesis was minimal (data not shown). Thus, 6-day-old cells were considered to be in G_0 phase. Cultures of these cells were grown in complete fresh medium at a concentration of 2.0×10^5/ml. They were harvested immediately and every 2 h thereafter, washed once with PBS and

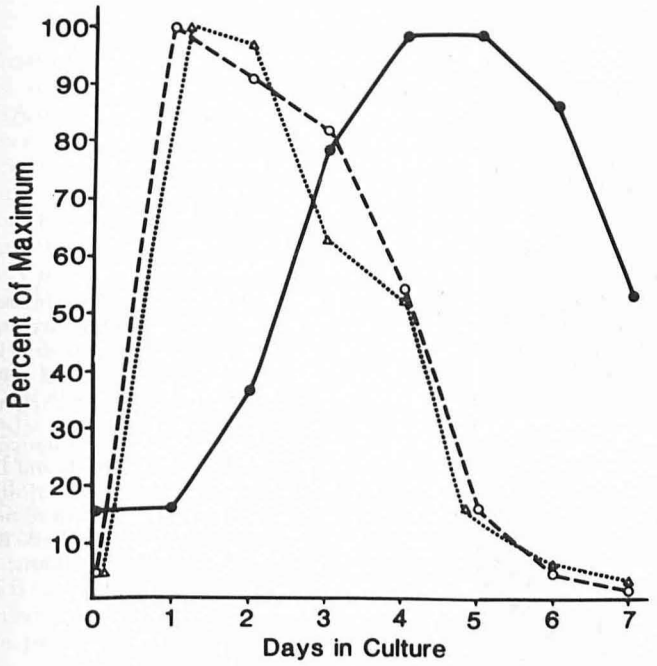

Figure 1. WiL2 cells were grown in suspension culture at 37°C. Daily cell counts were depicted by the growth curve (*solid circles*). Incorporation of [^{3}H]thymidine (*open circles*) and percentage of cells in DNA synthesis (labeling index, *triangles*) were determined by radioactivity counting (cpm) and autoradiography, respectively. By the sixth day of culture, less than 10% of the cells showed DNA synthesis. Also, less than 1% of these 6-day-old cells demonstrated mitotic activity (not shown). These were thus considered to represent the G_0 or resting phase.

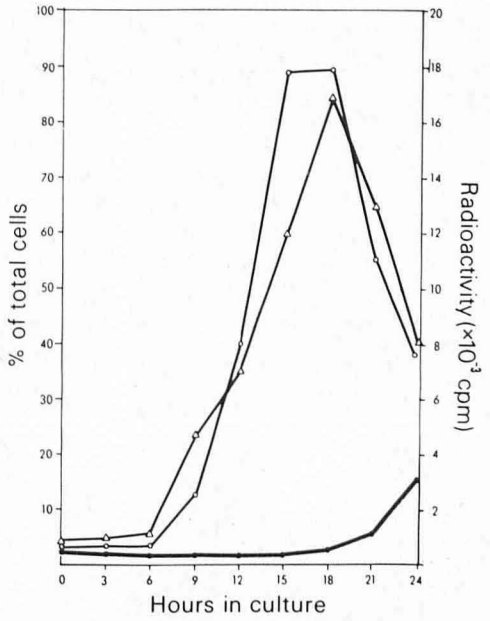

Figure 2. Determination of the cell cycle phases of WiL2 by density-dependent arrest. [^{3}H]Thymidine uptake (*triangles*), percentage of cells in mitosis (mitotic index, *solid circles*), and percentage of cells in DNA synthesis (labeling index, *open circles*), were determined every 3 h. In the first 6 h, greater than 90% of cells showed no DNA synthesis, but subsequently they entered DNA synthesis, reaching a maximum between 15–18 h. Following a rapid decline in these synthetic cells (S phase), significant numbers of mitotic cells appeared at 21–24 h.

cytocentrifuged onto glass microscope slides, and used for indirect immunofluorescence. Some cells were harvested every 3 h, pulse-labeled with [^{3}H]thymidine, and processed for autoradiography to determine thymidine uptake, labeling index, mitotic index as described above (Fig 2).

In the second method, WiL2 cells were arrested at the late G_1 or early S phase of growth using the hydroxyurea blockade [13]. In this method, 2-day-old cells in the log phase of growth were treated with 4 mM hydroxyurea for 17 h. The hydroxyurea was removed by washing and resuspending the cells in fresh, pre-warmed culture medium. Cells were harvested at 0 h and every 2 h thereafter, similar to the density-dependent arrest procedure.

Mixed Lymphocyte Cultures Blood was drawn from 2 healthy human subjects who had no symptoms of connective tissue disease. The lymphocytes from each subject were isolated using the Isopaque-Ficoll technique originally described in 1968 [14]. The blood was diluted twice with culture medium and the solution was carefully layered over 10 ml of Histopaque (Sigma Chemical Co., St. Louis, Missouri). The mixture was centrifuged at 2000 rpm for 15 min. The mononuclear cell-containing layer was clearly identified and removed using a pipet. Lymphocytes from each subject were divided. The first group was washed, resuspended in PBS, and cytocentrifuged onto glass slides (0 h). The remaining lymphocytes were mixed together and incubated at 37°C for 6 days. Labeling index and [^{3}H]thymidine uptake were also determined as described above. By 3–4 days of culture, some of the cells began showing DNA synthesis, and cells from the 4-day-old culture were harvested and used for indirect immunofluorescence (Fig 3).

Serum Specimens The human sera used in this study contained monospecific antibodies either to the SSA/Ro or SSB/La antigen. The monospecificity of these sera was based on the production of a single precipitin line on double immunodiffusion and counterimmunolectrophoresis using WiL2 cell extract as the antigenic source. In immunodiffusion, lines of immunologic identity were formed with standard reference sera, establishing the identity of the precipitating antibody. In addition, sera containing anti-SSA/Ro antibodies gave a negative fluorescent antinuclear antibody assay when mouse kidney section was used as substrate [15], but a speckled nuclear immunofluorescence pattern on human HEp-2 and WiL2 cells [15,16]. The anti-SSB/La antisera produced a speckled nuclear fluorescence pattern on both rodent tissue section and tissue culture cells. Other criteria included specific RNAs identified by immunoprecipitation, a specific peptide recognized in immunoblot analysis, and a negative radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) for DNA or histone antibody assays [9,17]. Four sera having anti-SSA/Ro antibody only were included in this study. Two anti-SSB/a sera that fulfilled the above criteria were selected and further subjected to SSA/Ro ELISA to prove no contamination of anti-SSA/Ro activity, and then used in this study.

SSA/Ro Enzyme-Linked Immunosorbent Assay SSA/Ro was purified from WiL2 cell extract by ammonium sulfate precipitation, polybuffer exchanger ion exchange chromatography, and followed by preparative 10% acrylamide electrophoresis as described by Lieu et al [18]. SSA/Ro was also purified by anti-SSA/Ro affinity column chromatography as described by Yamagata et al [19]. Purified SSA/Ro antigen was coated onto microtitration plates at the concentration of 0.1 μg/well and used for SSA/Ro ELISA. For ELISA, human sera were diluted to 1:100, 1:200, 1:400, and 1:800 with Tris-saline buffer, pH 7.4 containing 0.05% Tween and 1% bovine serum albumin. The diluted sera were added to microtitration plates and incubated at 37°C for 2 h. Following thorough washing in Tris-saline buffer with 0.05% Tween, horseradish peroxidase (HRP)-labeled goat antihuman IgG (1:1000 dilution)(Cappel Laboratory, Cochranville, Pennsylvania) was added and incubated for another 2 h. After washing, substrate was added and optical density reading

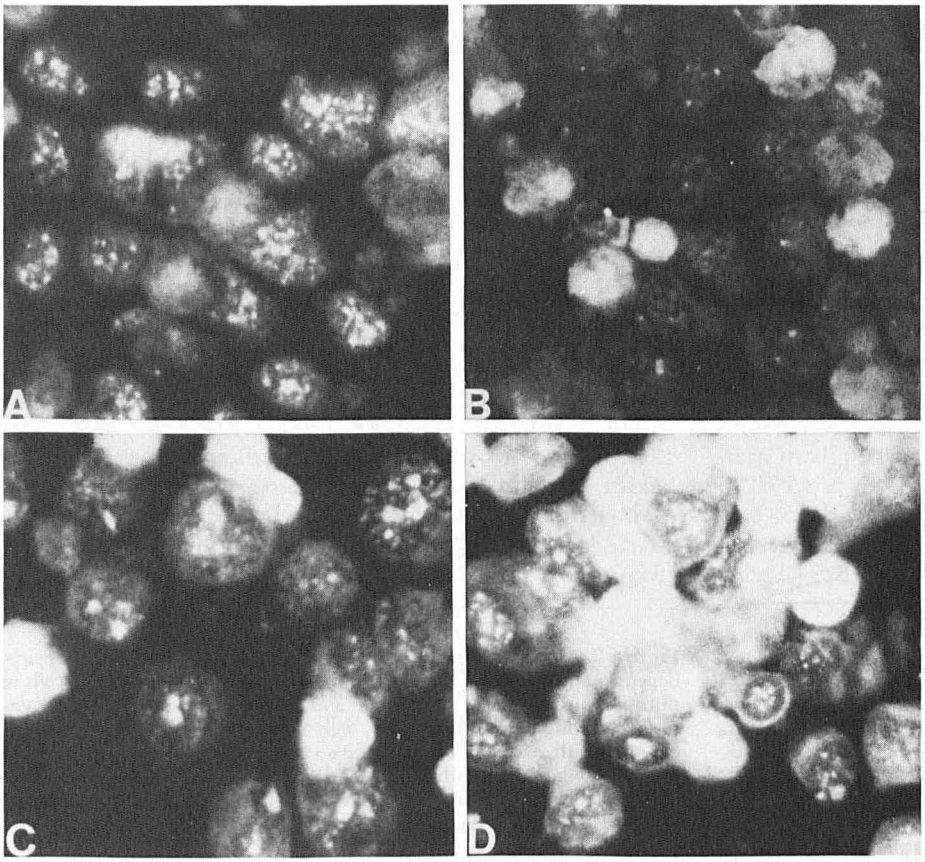

Figure 3. Fluorescent staining of synchronized WiL2 cells by the density-dependent arrest/-starvation method. Cells stained with anti-SSB/La are shown in (*A*) and (*C*), and those stained with anti-SSA/Ro in (*B*) and (*D*). *A and B*, Cells in G_0 phase: *A* = G_0 cells stained with SSB/La; *B* = G_0 cells stained with SSA/Ro. In both instances, one can see a nuclear speckled pattern. *C and D*, Cells in late G_1 and early S phase. SSB/La staining (*C*) reveals a prominent nucleolar pattern in addition to the nucleoplasmic speckles, but SSA/Ro staining (*D*) yields only the nuclear speckling.

at 405 nm using Dynatech Micro ELISA plate reader was undertaken.

Indirect Immunofluorescence The substrates used for immunofluorescence were WiL2 cells and human peripheral blood lymphocytes. Aliquots consisting of 0.1 ml of cell suspension were cytocentrifuged onto glass microscope slides and fixed in acetone for 10 min. Sera containing anti-SSA/Ro and anti-SSB/La antibodies were reacted with the substrate for 30 min and then washed in PBS for 5 min. The conjugate used was fluorescein isothiocyanate (FITC)-labeled goat antihuman IgG with the following characteristics: FITC, 132 μg/ml; protein, 14 mg/ml; antibody activity, 32 units/ml; plateau endpoint, 1:128 dilution. This antiserum was diluted 1:100 in PBS. The cells were incubated with conjugate for 30 min, then washed again for 5 min in PBS. The slides were counterstained with Evans blue, mounted with buffered glycerine, and read using a Zeiss epifluorescent microscope. Normal human serum or PBS was used as a negative control.

Purification of IgG and Labeling with FITC IgG was purified from monospecific anti-SSA/Ro and anti-SSB/La human antisera by ammonium sulfate salt precipitation and DE-52 ion exchange chromatography. The purified IgG was then coupled with FITC as described above [20]. Fluorescein isothiocyanate-conjugated specific IgG was then used for the competition experiments described in the following section.

Competition Experiments WiL2 cells were synchronized and harvested 8 h after initiation of the starvation synchronization. The cells were incubated for 30 min at room temperature with 1:5 dilution of anti-SSA/Ro IgG. This IgG was isolated from a monospecific anti-SSA/Ro antiserum, yielding a precipitin anti-SSA/Ro titer of 1:16. Following incubation, cells were washed in PBS and incubated with FITC-labeled anti-SSB/La IgG for 30 min. The cells were washed, mounted with glycerin, and examined using a fluorescent microscope. A second set was prepared using synchronized cells incubated with anti-SSB/La IgG followed by FITC-labeled anti-SSA/Ro IgG.

Table I. Characteristics of Anti-SSA/Ro and Anti-SSB/La Reference Sera

	Mouse	WiL2[a]	Precipitin Line	Immunoblot Analysis	RNA Profile	SSA ELISA A[b]	SSA ELISA B[c]
SSA/Ro 1[d]	Negative	Speckled	Single	60K	2 RNAs	0.475	0.615
2	Negative	Speckled	Single	60K	2 RNAs	0.546	0.714
3	Negative	Speckled	Single	60K	2 RNAs	0.593	0.610
4	Negative	Speckled	Single	60K	2 RNAs	0.415	0.496
SSB/La 1	Speckled	Speckled	Single	43K	6 RNAs	0.088	0.139
2	Speckled	Speckled	Single	43K	6 RNAs	0.105	0.156

[a] WiL2 cell smears.
[b] ELISA OD reading of serum dilution 1:100 using affinity-purified SSA/Ro ELISA plates. Normal human serum control <0.179.
[c] ELISA OD reading of serum dilution 1:100 using polybuffer exchanger ion exchange chromatography and acrylamide gel electrophoresis purified SSA/Ro ELISA plates. Normal human serum control <0.200.
[d] Serum number.

RESULTS

Monospecificity of the Reference Sera The characteristics of the sera used in this study are shown in Table I. All of the 4 anti-SSA/Ro sera were ANA-negative on mouse kidney sections, but gave a speckled nuclear staining pattern on WiL2 cell substrate [15,16], and had only 1 precipitin line in double immunodiffusion and counterimmunoelectrophoresis [9,17]. As shown before, they precipitated 2 small cellular RNAs in immunoprecipitation using ^{32}p-labeled WiL2 cell extract as the source of antigen. Moreover, they recognized only a 60,000-dalton peptide in immunoblot analysis [17]. Based on these criteria, it was quite certain that those anti-SSA/Ro sera were monospecific based on current available techniques. All these sera reacted in SSA/Ro ELISA. The 2 anti-SSB/La reference sera gave also only 1 precipitin line in counterimmunoelectrophoresis and double immunodiffusion, and were ANA-positive of speckled pattern, in mouse kidney sections and WiL2 cell smears. They recognized only a peptide of 43,000 daltons in immunoblot analysis [17]. Moreover, they precipitated 6 RNAs, 2 of which migrated to the same points as the RNAs precipitated by anti-SSA/Ro sera [17]. However, these 2 sera did not react in SSA/Ro ELISA. This indicated that these 2 anti-SSB/La sera did not contain detectable anti-SSA/Ro antibody activity and were assumed to be very monospecific and used in the present study.

Growth Curves Cell counts and viability of the WiL2 cell cultures were determined daily for 7 days and these results are shown in Fig 1. During the first 24 h, the cells were considered to be in the lag phase, while between day 1 and day 4, they entered the log or exponential growth phase. The cell count plateaued at days 4–5 and then began to decline (death phase). Viability (not shown) was maximal (90–95%) during cell growth, but started to decline on the fifth day.

Synchronization by Density-Dependent Arrest The results depicting [^{3}H]thymidine uptake, labeling index, and mitotic index are shown in Fig 2. In the first 6–9 h, most of the cells (90%) were in a phase with no DNA synthesis. At 9 h of culture, cells entered DNA synthesis, which reached a peak at about 18 h. Significant numbers of mitotic cells appeared at 24 h of culture. From these results, it was concluded that the first 9 h after release from density-dependent arrest represented the G_1 phase. S phase cells included those showing DNA synthesis (9–21 h), and G_2 and M phases were present at 21–24 h.

Immunofluorescent Staining Using monospecific serum for anti-SSB/La antibodies, immunofluorescent staining revealed a nuclear speckled pattern in the G_0 and G_1 phases as seen in Fig 3*A*. In late G_1 and early S phase (Fig 3*C*), there was strong nucleolar staining in greater than 90% of the cells in addition to the nucleoplasmic staining. During S phase, the speckled pattern remained, but the nucleolar staining had almost disappeared (not shown). The mitotic phase cells showed speckled staining surrounding chromosomes (not shown). The peak time for the nucleolar prominence was at 8 h, corresponding to late G_1 and early S phase.

Observations using antibody to SSA/Ro are also shown in Fig 3*B,D*. All gave speckled nucleoplasmic staining, but there was no evidence of nucleolar staining similar to that seen with anti-SSB/La antiserum. In addition, when both anti-SSA/Ro and anti-SSB/La antisera were serially diluted, only reduced staining intensity was noted without a change in staining pattern.

These results were confirmed by synchronization of the WiL2 cells with hydroxyurea block. Following release from the hydroxuyrea treatment, cells immediately entered the late G_1 and/or early S phase of the cycle. When these cells were stained with anti-SSB/La antiserum, they displayed a prominent nucleolar pattern with nuclear speckling. Staining with several monospecific sera for anti-SSA/Ro gave similar results, i.e., fine nuclear speckles without nucleolar staining.

The mixed lymphocyte culture (MLC) was used as an additional method to confirm the above observations. When lymphocytes from 2 genetically different individuals were mixed and cultured together, they underwent blast-transformation and proliferation. DNA synthesis began at 4 days of culture, shown in Fig 4. At 0 h prior to mixing the lymphocytes, immunofluorescence using PBS as a control revealed background and fine cytoplasmic staining. Examination of the MLC at 4 days coincided with the beginning of DNA synthesis or early S phase of the cell cycle. The control showed only background staining as on the 0 h smear and the SSB/La antiserum revealed a nucleolar as well as nuclear speckled pattern (Fig 5*A*). Anti-SSA/Ro sera elicited nucleoplasmic staining with some cytoplasmic staining, but failed to stain the nucleolus (Fig 5*B*).

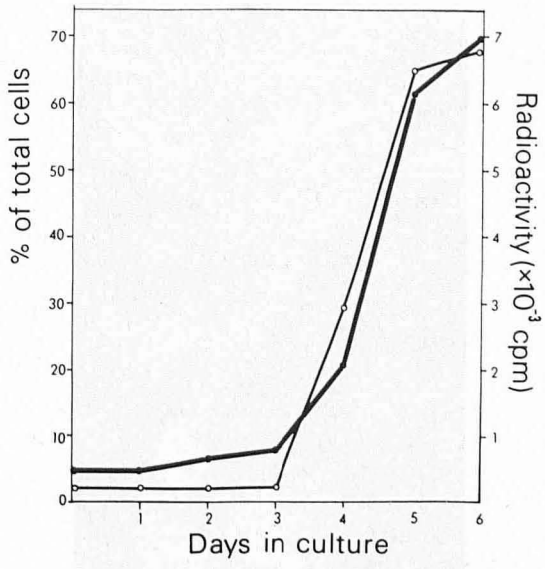

Figure 4. The mixed lymphocyte culture. At day 0 lymphocytes from 2 subjects were mixed and incubated. Increases in DNA synthesis (labeling index, *open circles*) and [^{3}H]thymidine uptake (*solid circles*) began at 3–4 days. The 4-day-old cells were considered to represent the beginning of the S phase.

Competition Experiments Synchronized cells of late G_1 and early S phase were incubated with anti-SSA/Ro IgG followed by FITC-labeled anti-SSB/LA IgG, or anti-SSB/La IgG followed by FITC-labeled anti-SSA/Ro IgG. There was nucleoplasmic and nucleolar speckled staining in cells treated with anti-SSA/Ro followed by FITC-labeled anti-SSB/La IgG. This corresponded to the original staining given by anti-SSB/La antisera in cells at late G_1 and/or S phase. However, there was no staining at all when the synchronized cells were stained first with anti-SSB/La followed by an FITC-labeled anti-SSA/Ro IgG.

DISCUSSION

The present investigation demonstrates that SSA/Ro and SSB/La cellular antigens are predominantly nuclear in location and show speckled or particulate staining patterns by fluorescence microscopy. These results confirm the reports of others [1,15–17,21,22]. In addition, we have illustrated how the SSA/Ro and SSB/La antigens are different at the cellular level. There are more SSB/La than SSA/Ro nuclear speckles microscopically, as expected from the relative cellular content of these antigens as described earlier by Lerner et al [23]. They estimated that each cell contains 10^6 copies of SSB/La but only 10^5 copies of SSA/Ro. We have also confirmed that the SSB/La molecule is a dynamic intracellular component, and have extended these observations in cells undergoing blastoform transformation during mixed lymphocyte reactions. SSB/La antigen is cell cycle-dependent, manifesting itself as a nuclear antigen with a nucleolar localization in late G_1

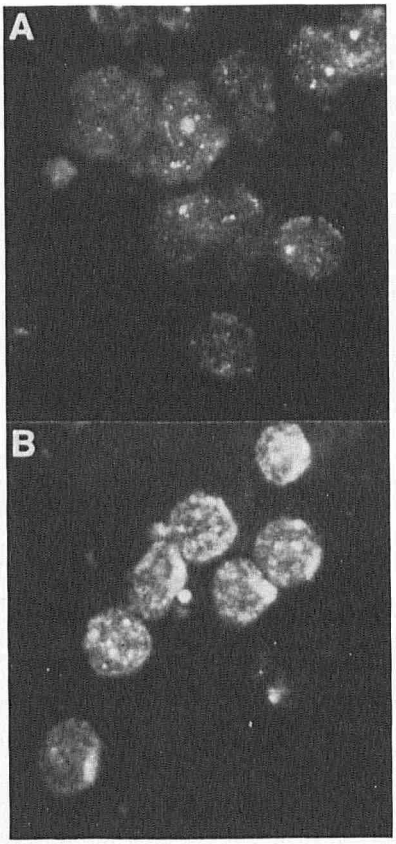

Figure 5. Staining patterns of cells in mixed lymphocyte culture. These photographs represent lymphocytes at 4 days of culture or the beginning of DNA synthesis. *A*, Prominent nucleolus and fine nuclear speckled pattern produced by anti-SSB/La antiserum. *B*, Nucleoplasmic speckled staining with SSA/Ro.

and early S phases (Figs 3*C*, 5*A*) [11], while SSA/Ro antigen is not cell cycle-dependent. This difference does not contradict the idea of a molecular relationship between SSA/Ro and SSB/La antigens [9,10]. It has been suggested that there are 2 distinct forms of SSB/La particles: free SSB/La and a ribonucleoprotein particle carrying SSB/La and SSA/Ro antigenic activities [10]. Both forms contribute to the nuclear speckled staining pattern in cells at G_0, early G_1, and late S phases, while only free SSB/La is responsible for the nucleolar staining pattern in cells at late G_1 and/or early S phase. This hypothesis is supported by our competition experiments, in which SSA/Ro antiserum did not block the nucleolar staining by anti-SSB/La antiserum in cells at late G_1 and/or early S phase. On the other hand, anti-SSB/La antiserum could effectively abolish the subsequent nuclear staining by anti-SSA/Ro antiserum. One possible explanation for these findings is contamination of anti-SSB/La activity by trace amounts of anti-SSA/Ro antibody. This is unlikely because the serum used was screened by strict criteria including a single peptide of 43K recognized in immunoblot analysis, a single precipitin line in counterimmunoelectrophoresis, the specific SSB/La associated RNA profile in immunoprecipitation [9,17], and the negative SSA/Ro ELISA. Future studies using monoclonal antibodies to SSB/La and SSA/Ro antigens must be performed to clarify this point.

The distribution of SSA/Ro antigen in cells is an interesting question. LeFeber et al reported the detection of SSA/Ro in cytoplasm as well as nucleus of cultured human keratinocytes [24]. The presence of SSA/Ro in cytoplasm was described initially by Clark and his associates in 1969 [25]. At that time, SSA/Ro was found in extract of cytoplasmic fraction of calf thymus and human spleen. Using monospecific human anti-SSA/Ro sera and purified anti-SSA/Ro antibody from immunodiffusion precipitin lines, Harmon and her associates [16] showed the intranuclear localization of SSA/Ro and, furthermore, they detected the presence of SSA/Ro in cytoplasm and nucleus, and even only in cytoplasm of cells fixed with methanol of varying duration. This suggests that SSA/Ro is primarily nuclear in location and the solubility of SSA/Ro antigen might contribute to its cytoplasmic location under certain conditions. Additionally, most of nuclear proteins are synthesized in cytoplasm and transported to nucleus rapidly. These 2 facts might explain the different localization of SSA/Ro antigen detected by different investigators. The most striking observation, however, is the cell membrane localization of SSA/Ro in some UVB-irradiated human keratinocytes described by LeFeber et al [24]. They described the dynamic nature of SSA/Ro antigen, present on cell membrane of viable, UVB-irradiated cultured human keratinocytes as well as in the cytoplasm and nucleus of UVB-irradiated permeabilized cultured human keratinocytes. If this is indeed a true phenomenon, it might explain the photosensitivity seen in patients with subacute cutaneous LE who have a higher frequency of positive anti-SSA/Ro antibody. This needs to be confirmed further by monoclonal antibodies.

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